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ABSTRACT

Buffalo milk is characterized by the presence of all 4 casein fractions (αS1, β, αS2, and κ) encoded by the 4 tightly linked autosomal genes (CSN1S1, CSN2, CSN1S2, and CSN3, respectively). In the present paper, we report for the first time a quantitative characterization of buffalo casein transcripts and show that the 4 genes are not transcribed and translated with the same efficiency. In particular, the analysis of individual milk samples obtained from 9 Mediterranean river buffaloes showed that the most abundant casein fractions were β (53.45%) and αS1 (20.61%), followed by αS2 and κ, at 14.28 and 11.66%, respectively. Quantification of the corresponding mRNA showed that the percentage of transcripts of the 4 caseins was 16.48, 23.18, 55.87, and 4.47% for αS1, β, αS2, and κ, respectively. Translation efficiency was 0.25 for CSN1S2, 1.31 for CSN1S1, 2.39 for CSN2, and 2.69 for the CSN3 transcripts, respectively. A comparison of nucleotide sequences with the Kozak consensus sequence was also carried out to investigate if the mRNA sequences might be responsible for the observed differences.

KEY WORDS: Mediterranean river buffalo , casein , mRNA , quantification

SHORT COMMUNICATION

Buffalo milk is characterized by the presence of all 4 casein fractions (αS1, β, αS2, and κ) encoded by 4 tightly linked autosomal genes (CSN1S1, CSN2, CSN1S2, and CSN3, respectively) mapped on chromosome 7 (Iannuzzi et al., 2003). Today, the complete amino acid sequences of buffalo casein (D'Ambrosio et al., 2008) are available, as well as the complete sequence and the relative regulatory regions of genes encoding β- (Cosenza et al., 2009a) and κ-casein (Masina et al., 2007), the 5'untranslated region (UTR), exon 1, and partial cDNA of the CSN1S1 gene (EMBL no. GU593719, AF529305, AY948385, and AJ005430), and the sequences related to the cDNA as well as short intronic sequences of the CSN1S2 gene (Cosenza et al., 2009b). Recently, Feligini et al. (2009) developed a method for the quantification of αS1-, β-, αS2-, and κ-caseins in water buffalo

milk using reverse-phase HPLC. Unlike what has been accomplished for cattle, sheep, and goat (Bevilacqua et al., 2006), no research has been carried out on the expression of casein genes in the buffalo species as well as on their translational efficiency. The aim of this study was therefore to assess the translation efficiency of the casein-encoding genes in Mediterranean river buffalo through analysis of protein abundance and mRNA gene levels. For this purpose, individual raw milk samples from 9 Mediterranean river buffaloes at comparable age, in third calving, at 120 d in milking, belonging to the same farm located in province of Salerno (Italy), and free of clinical mastitis were collected together with the monthly SCC controls by the local breeder association (ANASB, Caserta, Italy). After collection, samples were immediately frozen and kept at −20°C until analysis to prevent any proteolytic reaction induced by possible high SCC. Total RNA was extracted from somatic cells (SCC range from 10,000 to 12,000/mL) present in the fresh milk by using Nucleospin Blood and NucleoSpin Extract kits (Macherey-Nagel, Düren, Germany). The quantity, quality, purity, and integrity of RNA after DNase treatment were estimated by means of NanoDrop 2000c spectrophotometer (Thermo Scientific, Barrington, IL) and by electrophoresis on a denaturing agarose gel. The reverse transcription was performed using Improm-II Reverse Transcriptase (Promega, Madison, WI). By means of real-time quantitative PCR, the CSN1S1, CSN2, CSN1S2, and CSN3 mRNA were quantified using standard curves, and the amount of each transcript occurring within each sample was expressed as relative to the amount of transcript measured for the single internal control (18S rRNA) used for normalization. Primer sequences for CSN1S1, CSN2, CSN1S2, CSN3, and 18S rRNA amplification are reported in Table 1.

The results show that the transcript percentages were 16.48 (SD 4.99), 23.18 (SD 5.41), 55.87 (SD 8.22), and 4.47% (SD 0.96) for αS1, β, αS2, and κ, respectively. These values are significantly different from those characterizing the transcripts of the same genes in cattle, sheep, and goats. In fact, for these species, each casein transcript represents nearly 25% of the whole casein transcript population (Bevilacqua et al., 2006). Although the starting material is different (milk somatic cells in the present work and mammary gland cells in Bevilacqua et al., 2006), the results obtained by

different authors so far are comparable. In fact, different studies on goat and beef cows showed that the relative amount of milk protein mRNA in milk somatic cells and mammary tissue samples is highly correlated (Boutinaud et al., 2002; Murrieta et al., 2006).

The quantitative determination of the 4 casein fractions of the 9 individual buffalo milk samples was carried out according to the method of Feligini et al. (2009). In the examined samples, β- (19.81 g/L, SD 5.14) and αS1-casein (7.62 g/L, SD 2.52) were the major caseins, accounting for 53.45% (SD 6.63) and 20.61% (SD 4.29) of the whole casein fraction, respectively. On the contrary, αS2- (4.99 g/L, SD 0.92) and κ-casein (4.25 g/L, SD 1.05) were less abundant, at 14.28% (SD 4.88) and 11.66% (SD 2.26), respectively.

These results are in agreement with those found for buffalo by Feligini et al. (2009), but quite different from those obtained for cattle, sheep, and goat, which show a percentage distribution of β- and αS1-casein fractions of about 38% each (Bevilacqua et al., 2006). The observed differences in the percentage distributions of the 4 casein fractions in buffalo milk and, particularly, of the β-casein fraction, could account for its peculiar technological properties.

The ratio between the percentage of single milk protein fractions and the percentage of transcripts produced in the mammary gland was estimated in order to evaluate the translation efficiency of the buffalo gene casein transcripts. The values obtained show low translation efficiency (0.25, SD 0.07) for the CSN1S2 transcripts, whereas the efficiency was higher for the CSN3, CSN2, and CSN1S1 transcripts: 2.69 (SD 0.74), 2.39 (SD 0.49), and 1.31 (SD 0.30), respectively. These results disagree from those obtained by Bevilacqua et al. (2006) in cattle, goat, and sheep, in which the CSN2 and CSN1S1 mRNA showed higher translational efficiency.

A comparison of nucleotide sequences with the Kozak consensus sequence was accomplished to investigate if the mRNA sequences might be responsible for the observed differences. The context of the translation initiation codon (AUG) plays an important role in determining the translation rate (Kozak, 1991a,b). The Kozak consensus sequence occurs in eukaryotic mRNA and has the sequence GCCGCCRCCAUGG, where R is a purine (adenine or guanine) 3 bases upstream of the

start codon (AUG), which is followed by another G (Kozak, 1987). The A nucleotide of the AUG codon is referred to as number 1 and, although nucleotides −6, −3, and +4 are the most conserved positions in natural mRNA sequences, it seems likely that other nearby nucleotides also contribute to the translation process (Kozak, 1984; De Angioletti et al., 2004). In general, the more the sequence around the initiation codon is homologous to the Kozak sequence (i.e., "strong" consensus), the higher the efficiency of mRNA translation (Kozak, 1984).

The comparison of the sequence of the 4 transcripts in river buffalo with the Kozak sequence showed that CSN1S1 had 4 positions out of 6 conserved in the GCCRCC consensus motif, whereas CSN2, CSN3, and CSN1S2 had 3 homologous nucleotides (Table 2). In particular, 3 residues directly upstream of the initiation codon were consecutive in CSN1S1 and CSN2 (−3, −2, and −1) and 2 in CSN3 (−3 and −2). On the contrary, no conserved nucleotides were consecutive in CSN1S2 (−6, −3, and −1; Table 2), which could account for the lower translational efficiency recorded for the αS2-casein transcripts.

Concerning the higher translation efficiency of the κ-casein transcript compared with that observed by Bevilacqua et al. (2006) for cattle, sheep, and goat, an explanation could be found through comparison of the homologous messenger sequence among these species. The CSN3 mRNA in buffalo, cattle, sheep, goat, mouse, rabbit, and pig have 2 consecutive AUG and, with the exception of buffalo, in all these species, the first start codon shows a guanine in position −3 (Table 3).

It was proven (Kozak, 1984) that a start codon flanked by A in position −3 compared with G works considerably better; that is, has higher translational activity. Therefore, the initiation sites may also be designated as "strong" or "weak" based on this position. Ribosomes will initiate at the first AUG codon to a limited extent even when the context is weak, but the poor context allows some ribosomes to bypass the first AUG and reach a start codon further downstream, thus increasing the complexity level of an organism by alternative gene expression pathways. This is called leaky scanning (Kozak, 1984, 2005). Therefore, the presence of A in position −3 in the first start codon

could represent the optimal situation to ensure a more accurate and efficient translation of the buffalo CSN3 transcript compared with that in other ruminants.

In conclusion, we report here for the first time a quantitative characterization of the buffalo casein transcripts and show that the 4 mRNA are not transcribed and translated with the same efficiency. Although the analysis of the sequences flanking the start codon can help to formulate hypotheses concerning some of the observed differences in translation efficiency of the transcripts of the investigated genes, other elements need to be analyzed to fully understand the mechanisms of regulation of their expression. Based on results of different studies in higher eukaryotes and yeast, it is generally accepted that not only the context of the translation initiation codon (AUG), but also the length or alteration of the 5c UTR, secondary structure, GC content, upstream AUG codons or upstream open reading frames, and the length of the 3c UTR play important roles in determining the translation rate (Kozak 1991a,b; Tanguay and Gallie, 1996; Morris and Geballe, 2000; Koda et al., 2004).

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184 Table 1. Oligonucleotide primers sequence and positions

Gene	Position, nucleotides	Primer sequence (5′ to 3′)[1]		EMBL accession no.	Amplicon size, bp
CSN1S1	867–886	Forward	TCTTCTTGAGTTCTCTACTG	AY948385	114
	Complementary to 963–980	Reverse	ACTCAGTGGCCTTTATAC		
CSN2	126–144	Forward	AAGCCTTTCAAGCAGTGAG	FM946182	68
	Complementary to 174–193	Reverse	CCTCACTCTGAAACTTCTCA		
CSN1S2	574–591	Forward	AAAATCAGCCAGCATTAC	FM865618	111
	Complementary to 666–684	Reverse	GGGAATAACGTTTGTCTTA		
CSN3	14100–14117	Forward	TCAGTGAACAGAGAATAT	AM900443	106
	Complementary to 14189–14205	Reverse	GCTTTATTATGCAGGAA		
18S rRNA	1337–1352	Forward	CGTTCTTAGTTGGTGG	NR_036642	76
	Complementary to 1396–1412	Reverse	GTAACTAGTTAGCATGC		

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186 [1]Primers were designed by means of Oligo 5.0 software (National Biosciences Inc., Plymouth, MN).

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Table 2. Comparison of start codon flanking sequences of the 4 casein transcripts in the Mediterranean river buffalo

	Position[2]									
Sequence[1]	−6	−5	−4	−3	−2	−1	+1	+2	+3	+4
Kozak consensus sequence	G	C	C	R	C	C	A	U	G	G
CSN2	A	G	A	**G**	**C**	**C**	**A**	**U**	**G**	A
CSN1S2	**G**	U	A	**A**	A	**C**	**A**	**U**	**G**	A
CSN1S1	A	**C**	A	**A**	**C**	**C**	**A**	**U**	**G**	A
CSN3	**G**	G	U	**A**	**C**	A	**A**	**U**	**G**	A

[1]Kozak consensus sequence = the optimal context for initiation of translation in mammals. CSN2, CSN1S2, CSN1S1, and CSN3 are the genes encoding β-, αS2-, αS1-, and κ-casein, respectively.

[2]The start codon (AUG) in the 4 casein transcripts is underlined; conserved nucleotides are shown in boldface.

196 Table 3. Comparison of start codon flanking sequences of the κ-casein transcripts in different

197 species

Species	EMBL accession no.	Position[1] −6	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6	+7
Buffalo	AM900443	G	G	U	A	C	A	A	U	G	A	U	G	A
Cattle	AY380229	G	G	U	G	C	A	A	U	G	A	U	G	A
Sheep	NM_001009378	G	G	U	G	C	A	A	U	G	A	U	G	A
Goat	X60763	G	G	U	G	C	A	A	U	G	A	U	G	A
Pig	NM_001004026	G	G	U	G	C	A	A	U	G	A	U	G	A
Rabbit	Z18243	G	G	U	G	C	A	A	U	G	A	U	G	A
Rat	NM_007786	G	G	U	G	C	A	A	U	G	A	U	G	A

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199 [1]The first of 2 consecutive AUG codons is underlined.